p < 0.001). Among children under two years of age, PCV13 uptake increased from 2011 to 2012 (Table 1). Antibiotic consumption in the month preceding sampling was higher in the urban area. Pneumococcal carriage ranged between 60% and 65% in both regions and years.

Table 1. Characteristics of the two regions in both years.

	Urban area		Rural area	
	2011 %	2012 %	2011 %	2012 %
≥1 dose of any PCV	78.6	74.7	83.7	88.0
≥1 dose of PCV13 among children ≤ 2 years	50.8	67.6	65.3	79.7
Antibiotic consumption	20.9	20.8	9.1	4.8
Pneumococcal carriage	63.6	60.8	60.9	65.3

A lower percentage of VTs was observed in the rural area when compared to the urban area (18.1% *vs*. 23.7%, p = 0.054).

Furthermore, the six PCV13 additional serotypes (1, 3, 5, 6A, 7F and 19A) were mainly found among children who had not received PCV13 (24.4% *vs.* 12.8% in 2011, p = 0.027, and 27.6% *vs.* 6.6% in 2012, p < 0.001).

In the urban area serotypes 21, 19F, 10A, 6C and 15B/C were the most prevalent in 2011, whereas serotypes 23B, NT, 6C, 3 and 35B were the most prevalent in 2012.

In the rural area serotypes 11A, 23A, 22F, 24F and 6C were the most prevalent in 2011, and serotypes 15B/C, 31, 23B, 15A and 3 were the most prevalent in 2012.

Around 80% of all isolates were susceptible to the antibiotics tested in both years. Regarding the resistant isolates, these were mostly non-vaccine types (58.1% in 2011 and 72.2% in 2012).

4. Conclusion

PCV13 is impacting on colonization and the six additional vaccine-types (1, 3, 5, 6A, 7F and 19A) were mostly detected in non-immunized children. Further studies are needed in the upcoming years to monitor and unequivocally establish the impact of PCV13 in colonization.

Acknowledgments

Laboratórios Pfizer, Lda. and Fundação para a Ciência e a Tecnologia (PTDC/SAU-ESA/65048/2006 and Pest-OE/EQB/LA0004/2011).

References

[1] Raquel SÁ-LEÃO, Sónia NUNES, António BRITO-AVÔ, Carla ALVES, João A. CARRIÇO, Joana SALDANHA, Jonas S. ALMEIDA, Ilda SANTOS-SANCHES, Hermínia DE LENCASTRE. *High rates of transmission of and colonization by* Streptococcus pneumoniae *and* Haemophilus influenzae *within a day care center revealed in a longitudinal study*, J Clin Microbiol, 46, 225-34, 2008.

Influence of the capsular polysaccharide in biofilm formation by *Streptococcus pneumoniae*

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ABSTRACT:

The vast majority of infections are caused by microorganisms grown as biofilms. Recent evidences from several laboratories have demonstrated that both *in vivo* and *in vitro* formation of pneumococcal biofilms are hindered by the presence of capsular polysaccharide. We have analyzed the biofilm-forming capacity of clinical pneumococcal isolates of various serotypes. Strains of serotypes 19A and 19F, but not 19B and 19C, formed ≥80% of the biofilm of the nonencapsulated control strain. Strains of serogroup 6 also showed a significant biofilm-forming capacity. Serotypes 19A and 19F and serogroup 6 have in their structures the disaccharides α-D-Glcp-(1→2)-α-L-Rhap-(1→ and α-D-Glcp-(1→3)–α-L-Rhap-(1→. Serotypes 18A and 18C have a very similar disaccharide: α-D-GlcpNAc-(1→3)-β-L-Rhap-(1→ and α-D-Glcp-(1→3)-β-L-Rhap-(1→, respectively; however, pneumococcal strains of these serotypes showed impaired biofilm formation. Our results indicate that the chemical composition/structure of the capsular polysaccharide is crucial to define the biofilm-forming capacity of a particular *S. pneumoniae* isolate.

Keywords: capsular polysaccharide, biofilm formation.

1. Introduction

The pneumococcal population has changed since the widespread introduction of PCV7, directed at 7 (4, 6B, 9V, 14, 18C, 19F, and 23F) of the 94 pneumococcal capsular serotypes known to date. Non-PCV7 serotype isolates have increased among asymptomatic carriers and, to a lesser extent, non-PCV7 serotype pneumococci have increased as causes of invasive pneumococcal disease [1]. Hence, an increase in the incidence of infections caused by multiresistant 19A pneumococci has been observed [2].

It is now widely recognized that the vast majority of infections are caused by microorganism grown as biofilms. A biofilm is defined as a thin layer of microorganisms that adhere to the surface of an organic or inorganic structure embedded in an extracellular matrix [3]. Recent evidences from several laboratories have demonstrated that both *in vivo* and *in vitro* formation of pneumococcal biofilms is hindered by the presence of capsular polysaccharide (CPS) [4] and that exists an inverse relationship between the ability of the nonencapsulated variants to form biofilms and the amount of CPS produced [5].

To gain further information on the role of CPS in the initial steps of biofilm formation, we have analyzed the capacities of clinical pneumococcal isolates of various serotypes to develop biofilms. In contrast with the previous belief, some of the clinical serotypes analyzed are good biofilm formers, which may explain its prevalence in the human nasopharynx.

2. Results

The analysis of biofilm-forming capacity of various serogroup 19 pneumococcal isolates showed that most strains of serotypes 19A and 19F form ≥80%

of the biofilm of the control, nonencapsulated strain M11. In contrast, strains belonging to serotypes 19B and 19C showed a drastic reduction in the number of biofilm-associated sessile cells. Similar results were observed with the corresponding isogenic capsular transformants producing serogroup 19 capsules, generated to reduce the genetic variability among strains.

A close comparison of the primary structures of the repeating units of the CPS of types 19F/A on one hand, and 19B/C on the other, suggested that the disaccharides α-D-Glcp-(1→2)-α-L-Rhap-(1→ and α-D-Glcp-(1→3)–α-L-Rhap-(1→ (present in serotypes 19F and 19A CPSs, respectively, but not in the 19B and 19C capsules) may be important for promoting biofilm formation in the pneumococcus.

The disaccharide α-D-Glcp-(1→3)-α-L-Rhap-(1→ is also present in the CPS of the four serotypes of the serogroup 6. Previous studies of the biofilm-forming capacity showed that serotype 6B isolates formed 70% of the biofilm of strain M11 [6]. The analysis of others members of this group, namely serotypes 6A and 6C, showed that both, clinical and M11 transformants expressing these capsules, were able to form more than 50% of the biofilm of the nonen-capsulated control strain.

Biofilm-formation capacity was also studied in serotypes 18A and 18C. Serotype 18C has a glycerol-phosphate substituent that must be preserved for conserving the adequate anti-genicity of the 18C capsular polysaccharide [7]. Serotypes 18A and 18C have in their structure the disaccharides α-D-GlcpNAc-(1→3)-β-L-Rhap-(1→ and α-D-Glcp-(1→3)-β-L-Rhap-(1→, respectively, very similar to the ones mentioned above. However, a single isolate of either serotype able to form a significant biofilm was not found.

3. Conclusion

The results presented in this study indicate that, in addition to its genetic background, the chemical composition/structure of CPS is crucial to define the biofilm-forming capacity of a particular *S. pneumoniae* isolate.

Acknowledgements

The authors thank E. Cano for skillful technical assistance. This work has been sponsored by a grant from the Dirección General de Investigación Cien-tífica y Técnica (SAF-2009-10824). CIBER de Enfermedades Respiratorias (CIBERES) is an initiative of Instituto de Salud Carlos III (ISCIII).

References

[1] DM WEINBERGER et al., *Prediction of serotypes causing invasive pneumococcal disease in unvaccinated and vaccinated populations,* Epidemiology, 22, 199–207, 2011.

[2] A FENOLL et al., *Increase in serotype 19A prevalence and amoxicillin non-susceptibility among paediatric* Streptococcus pneumoniae *isolates from middle ear fluid in a passive laboratory-based surveillance in Spain, 1997–2009*, BMC Infect. Dis., 11, 239, 2011.

[3] JW COSTERTON *et al., Microbial biofilms*, Annu. Rev. Microbiol., 49, 711–745, 1995.

[4] M DOMENECH, E GARCÍA, M MOSCOSO, *Biofilm formation in* Streptococcus pneumoniae, Microb. Biotechnol., 5, 455–465, 2012.

[5] DOMENECH M, GARCÍA E, MOSCOSO M., *Versatility of the capsular genes during biofilm formation by* Streptococcus pneumoniae, Environ. Microbiol., 11, 2542–2555, 2009.

[6] MOSCOSO M, GARCÍA E, LÓPEZ R. *Biofilm formation by* Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accre- tion*, J. Bacteriol., 188, 7785–7795, 2006.

[7] J CHANG *et al., Relevance of O-acetyl and phosphoglycerol groups for the antigenicity of* Streptococcus pneumoniae *serotype 18C capsular polysaccharide,* Vaccine, 30, 7090–7096, 2012.

Insight into the composition of the intercellular matrix of *Streptococcus pneumoniae* biofilms

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ABSTRACT:

Biofilm matrices consist of a mixture of extracellular polymeric substances synthesized in large part by the biofilm-producing microorganisms themselves. These matrices are responsible for the cohesion and three-dimensional architecture of biofilms. The present study demonstrates the existence of a matrix composed of extracellular DNA, proteins and polysaccharides in the biofilm formed by *Streptococcus pneumoniae*. Extracellular DNA, visualized by fluorescent labeling, was an important component of the matrix. The existence of DNA–protein complexes associated with bacterial aggregates and other polymers was hypothesized based on the unexpected DNA binding activity of lysozyme LytC. The presence of intercellular DNA-LytC protein complexes in pneumococcal biofilms was demonstrated by confocal laser scanning microscopy. Evidence of extracel-lular polysaccharide different from the capsule was obtained by staining with Calcofluor dye and four types of lectin conjugated to Alexa fluorophores, and by incubation with glycoside hydrolases. The presence of residues of Glcp(1,4) and GlcNAc(1,4) in the pneumococcal biofilm was confirmed by GC-MS techniques.

Keywords: biofilm, matrix composition, eDNA, polysaccharide, EPS.

1. Introduction

Recent reports have shown the in vivo for-mation of *S. pneumoniae* biofilms on adenoid and mucosal epithelial tissues in children with recurrent or chronic ear infections (for a recent review, see ref. 1). Using low-temperature scanning electron microscopy (LTSEM) techniques, we reported a biofilm matrix containing an intercellular, fiber-like material that linked the pneumococcal cells to one another and to the glass substrate on which they were grown [2]. The presence of extracellular proteins in this matrix was inferred from the biofilm-disaggregating activity of proteolytic enzymes [2].

Several authors have reported extracellular DNA (eDNA) to be an important extracellular polymeric substance (EPS) in pneumococcal biofilms, based on the dramatic disappearance and inhibited formation of biofilms following treatment with DNase [2].

Controversy exists over whether extracellular polysaccharide is a component of the *S. pneumoniae* biofilm matrix. Using encapsulated *S. pneumoniae* cells to demonstrate the existence of a polysaccharide among the EPS is, however, problematic since some lectins also bind the capsular polysaccharide, as demonstrated more than 30 years ago [3].

The present work provides evidence that the biofilm formed by *S. pneumoniae* R6 contains a matrix made up of eDNA, protein and polysaccharide components.